# ON THE MECHANISM OF PHOSPHATE AND DICARBOXYLATE TRANSPORT IN MITOCHONDRIA

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## 1. Introduction

Studies on the swelling of mitochondria in iso-osmotic solutions of the ammonium salts of phosphoric acid and of various dicarboxylic and tricarboxylic acids, led to the postulation of 3 functionally-related but separate anion transporters in the inner mitochondrial membrane [1,2]: a phosphate/$OH^-$, a phosphate/dicarboxylate and a dicarboxylate/tricarboxylate antiporter. In further studies [3–6], using the swelling technique [7–9], or more direct methods to study the transport of anions [10–15], the main conclusions were further substantiated (reviewed [17–21]).

Although the 2 transport systems for phosphate could be distinguished by the discovery of the specific inhibitors *N*-ethylmaleimide for phosphate/$OH^-$ exchange [5,11] and butylmalonate for phosphate/dicarboxylate exchange [3,11], experiments on the effect of the 2 inhibitors on mitochondrial phosphate and dicarboxylate exchanges, led to the proposition that the inner mitochondrial membrane contains only 1 carrier for phosphate and dicarboxylates [22,23]. Kinetic analysis by a modified Hill-plot of the effect of *N*-ethylmaleimide and butylmalonate on $^{32}P$ uptake in mitochondria clearly demonstrated a mutual interdependence of both inhibitor binding sites [24]. In an investigation on the induction of mitochondrial swelling in $NH_4$-malate by the phosphate analogues thiophosphate, mono- and di-fluorophosphate, we concluded that the uptake of malate into mitochondria does not occur via an exchange with phosphate, but by activation of the transporter by phosphate [25].

This paper presents striking evidence, that the previous interpretation of the induction of mitochondrial swelling in $NH_4$-malate by phosphate [2] was incorrect. Instead it is shown that phosphate acts from outside, without being transported. This conclusion is based on the effect of *n*-octylphosphate on malate transport in rat liver mitochondria.

## 2. Materials and methods

Di-Tris-*n*-octylphosphate was prepared as in [26] by reaction of *n*-octanol with $POCl_3$. Subsequent hydrolysis gave the free acid, which was converted to the Tris-salt [29]. For stability of phosphate esters see [27]. *N*-Ethylmaleimide was obtained from Serva (Heidelberg). Other chemicals were of analytical grade.

Isolation of mitochondria and swelling experiments were performed as in [25,28]. The iso-osmotic swelling media contained: 80 mM di-Tris-*n*-octylphosphate (prepared fresh) or phosphate, 40 mM $NH_4Cl$, pH 7.3; and 80 mM sodium malate, 40 mM $NH_4Cl$, 10 mM imidazol, pH 7.3.

## 3. Results and discussion

In previous studies *n*-octylphosphate was found to inhibit phosphate uptake in rat liver mitochondria competitively, as shown by the Ca-acetate-swelling, phosphate-shrinking method [29]. In fig.1 the swelling of rat liver mitochondria in iso-osmotic solution of $NH_4$-phosphate and of $NH_4$-*n*-octylphosphate is compared. Whereas a large and rapid swelling is obtained

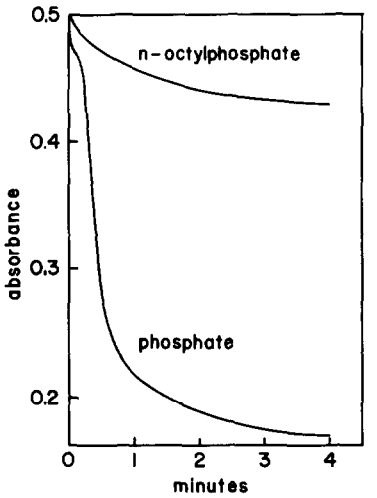


Fig.1. Swelling of rat liver mitochondria in iso-osmotic solutions of $NH_4$-phosphate and $NH_4$-*n*-octylphosphate. For composition of swelling media see section 2.

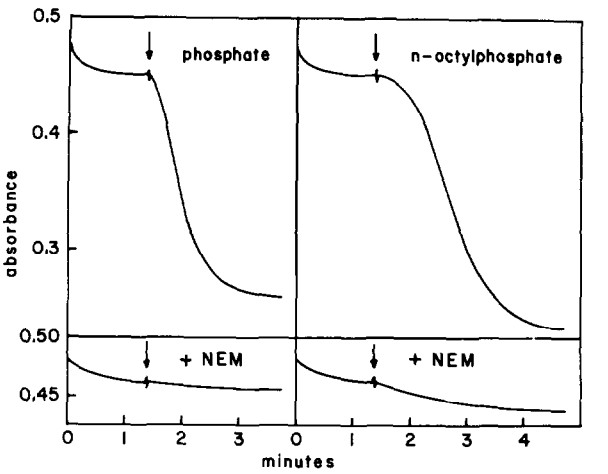


Fig.2. Induction of swelling of rat liver mitochondria in iso-osmotic solution of $NH_4$-malate by phosphate and *n*-octylphosphate. Phosphate and *n*-octylphosphate were added at the indicated time at final conc. 0.6 mM. Preincubation of mitochondria with *N*-ethylmaleimide (85 nmol/mg mitochondrial protein) was performed for 2 min at 0°C.

with phosphate, no corresponding swelling is obtained with *n*-octylphosphate. This is not surprising, since the phosphate carrier is expected to be rather specific. It should be mentioned that the small decrease of absorbance with time, in the case of *n*-octylphosphate, is not due to an uptake, since the same small decrease is also obtained in the presence of mersalyl, or with other non-permeable isotonic salt solutions.

The swelling of mitochondria in iso-osmotic $NH_4$-malate is shown in fig.2. Only after addition of 0.6 mM phosphate a large and rapid swelling is obtained as first described [2]. However, 0.6 mM *n*-octylphosphate could also induce the swelling of mitochondria in iso-osmotic $NH_4$-malate, with a similar rate and amplitude. Although the swelling curves were induced with low concentrations, it could not be excluded that *n*-octylphosphate, due to the long hydrophobic chain, might change the membrane permeability unspecifically. Therefore the effect of *N*-ethylmaleimide, a rather specific inhibitor of the phosphate transport in mitochondria, which does not inhibit the dicarboxylate transport [11], was studied. As can be seen in fig.2, the uptake of malate in mitochondria, induced by both phosphate and *n*-octylphosphate, was completely inhibited by *N*-ethylmaleimide.

This result is not compatible with an uptake of

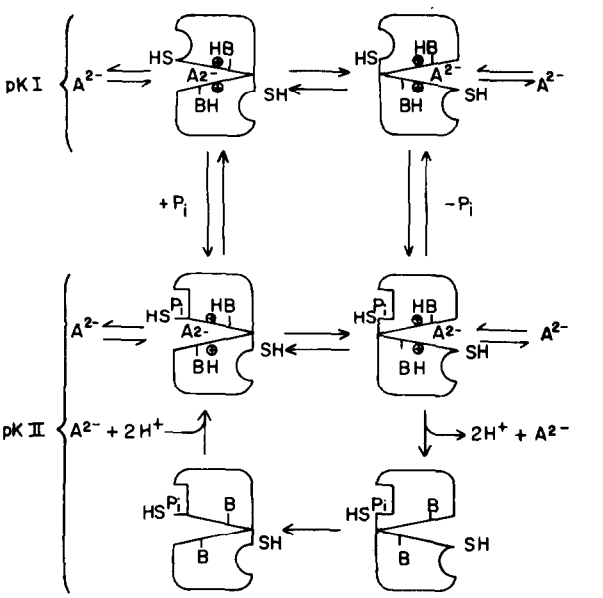


Fig.3. Model of the phosphate–dicarboxylate transport system of mitochondria. B, base at the transporting binding site, $BH^+ \rightleftharpoons B + H^+$; p*K* I (antiporter, absence of phosphate) > pK II (symporter, presence of phosphate); $A^{2-}$, divalent anion ($HPO_4^{2-}$, $malate^{2-}$, $succinate^{2-}$).

malate via an exchange with phosphate or *n*-octylphosphate, since the latter cannot be taken up by the mitochondria (fig.1).

On the other hand, it clearly indicates that induction of malate uptake in mitochondria by phosphate and by *n*-octylphosphate, is mediated by the same *N*-ethylmaleimide-sensitive mechanism. This uptake must be induced by phosphate or *n*-octylphosphate from the outer side and can only be interpreted as a malate/proton symport or, less probable, as a malate/hydroxyl antiport.

In order to understand these findings, we present a new model on the phosphate and dicarboxylate transport system in mitochondria, which can easily explain our results and most of the data from the literature (fig.3). The model includes the following characteristics:

1. Phosphate and dicarboxylates are transported in mitochondria by the same transport system.

2. Phosphate acts on the transporter as allosteric effector by decreasing the p*K* of the base at the transporting binding site from a higher (anion$^{2-}$/ anion$^{2-}$ antiporter) to a lower value (anion$^{2-}$/ 2 H$^{+}$ symporter).

3. In both conformations the transporter is able to exchange divalent anions. But only in the symporter conformation phosphate or dicarboxylates can be taken up actively, driven by a pH gradient.

4. *N*-Ethylmaleimide reacts with a SH-group at the allosteric binding site for phosphate, thus preventing the conversion of the antiporter into the symporter.

This model does not define the number and location of different allosteric binding sites for phosphate or other effectors, and cannot say whether the transporting binding sites for phosphate and dicarboxylate are identical or not.

The model, however, can easily explain different lag-phases observed in the induction of malate swelling of mitochondria by phosphate analogues [25] or *n*-octylphosphate (fig.2), since the rate of the postulated conformational change of the transporter should depend on the structural similarity of the analogue to phosphate. In addition the model implicates a regulation of substrate and phosphate uptake dependent on the pH difference between the intra- and extra-mitochondrial compartment. An increased ratio of distribution of phosphate and malate between the inner and outer compartment of mitochondria with decreased pH was observed [30] and it was suggested 'that all these substances are translocated associated with an equivalent amount of $H^{+}$' [30].

In accordance with the model is also the described protection of phosphate transport by phosphate against inhibition by *N*-ethylmaleimide [9,31]. Conformational changes induced by binding of phosphate to the allosteric site may change the accessibility of the SH-group. Conformational changes of a mitochondrial anion transporter due to external or internal binding of substrates is not new. From kinetic analysis of the oxoglutarate carrier of rat heart mitochondria it was concluded [32,33] that substrate binding on external binding sites will cause cooperative effects on the exchange rate.

Finally the model should be understood as a working hypothesis which may be further developed or withdrawn by future experiments.

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